APPENDIX - IV

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Format for Outline of Proposed Research work
(to be supplied in Six copies)

1. Name of Scholar (In English): Nilima Nimawat

(In Hindi): नीलिमा निमावत

2. Title of the Research Work:

(In English): **"Assessment of Genetic diversity and antioxidant potential in Drumstick (*Moringa oleifera* Lam.)"**

(In Hindi): ड्रमस्टिक (मोरिगां ओलीफेरा लेम) में अनुवांशिक विविधता एवं एन्टीऑक्सिडेन्ट क्षमता का मुल्यांकन

3. Location

a. Institution/Department where the work is to be done: Department of Botany
S.P.C Government College, Ajmer

b. Geographical area of Investigation, if any: Ajmer and surrounding area

4. Introduction Importance of Proposed Investigation, Objectives.

Introduction

The *Moringa oleifra* Lam. or Drumstick is a monogeneric genus belongs to the family Moringaceae family having 13 species of which *M. oleifera* has so far become the most used and studied. This species is a fast growing soft wood tree that can reach 12 m in height and is indigenous to the Himalayan foothills of northern India, Pakistan and Nepal (Sharma,V,et.al, 2011 and Roloff, A. et al, 2009). Its multiple uses and potential attracted the attention of farmers and researchers in past historical eras. Ayurvedic traditional medicine says that *Moringa oleifera* can prevent 300 diseases and its leaves have been exploited both for preventive and curative purposes (Ganguly, S. 2013).

M. oleifera is a fast-growing, deciduous tree. It can reach a height of 10–12 m (32–40 ft) and the trunk can reach a diameter of 45 cm (1.5 ft). The bark has a whitish-grey colour and is surrounded by thick cork. Young shoots have purplish or greenish-white, hairy bark. The tree has an open crown of drooping, fragile branches and the leaves build up a feathery foliage of tri-pinnate leaves.

The flowers are fragrant and bisexual, surrounded by five unequal, thinly veined, yellowish-white petals. The flowers are about 1.0-1.5 cm (1/2") long and 2.0 cm (3/4") broad. They grow on slender, hairy stalks in spreading or drooping later flower clusters which have a length of 10–25 cm. Flowering begins within the first six months after planting. In seasonally cool regions, flowering only occurs once a year between April and June. In more constant seasonal temperatures and with constant rainfall, flowering can happen twice or even all year-round.[6] The fruit is a hanging, three-sided brown capsule of 20–45 cm size which holds dark brown, globular seeds with a diameter around 1 cm. The seeds have three whitish papery wings and are dispersed by wind and water.

Importance of proposed investigation

All plant parts of *Moringa oleifera* are traditionally used for different purposes, but leaves are generally the most used (Popoola, J.O.and Obembe, O.O. 2013 and Sivasankari, B. et al. 2014). In particular, they are used in human and animal nutrition and in the traditional medicine. Leaves are rich in protein, mineral, beta-carotene and antioxidant compounds. In traditional medicine, these leaves are used to treat several ailments including malaria, typhoid fever, parasitic diseases, arthritis, swellings, cuts, diseases of the skin, genito-urinary ailments, hypertension and diabetes. They are also used to elicit lactation and boost the immune system (Sivasankari, B. et al. 2014; Kasolo, J.N. et al, 2010) , as well as cardiac stimulants and contraceptive remedy.

Moringa seeds have an oil content of 30%–40%, with a high-quality fatty acid composition *i.e.*, high oleic acid (Rashid, U. et al, 2008). In addition they posses significant resistance to oxidative degradation. These proprieties make Moringa oil a good candidate to produce biodiesel after transesterificaton (Rashid, U. et al, 2008; Da Silva, et al, 2010 and Ofor, M.O. 2011). Biswas and John 2008 , reported that approximately 3030 kg of oil are required to produce 1000 liters of biodiesel. Moringa seed powder can be used for water purification, replacing dangerous and expensive chemicals such as aluminum sulfate (Popoola, J.O.and Obembe, O.O. 2013)

Several bioactive compounds were recognized in the leaves of *Moringa oleifera*. They are grouped as vitamins, carotenoids, polyphenol, phenolic acids, flavonoids, alkaloids, glucosinolates, isothiocyanates, tannins, saponins and oxalates and phytates. The amounts of different bioactive compounds found in *Moringa oleifera* leaves.

Moringa oleifera dried leaves are a great source of polyphenols. These amounts of polyphenols are greater than those found in fruits and vegetable (Brat, P. et al, 2006; Fu, L. 2011; Kumar,S. et al, 2013). Principal polyphenol compounds in *Moringa oleifera* leaves are flavonoids and phenolic acids. *Moringa oleifera* leaves are a rich source of antioxidant compounds [114]. Hypoglycemic effects of Moringa oleifera leaves are reported in literature [132]. Experimental evidences showed the capacity of *Moringa oleifera* leaves to protect organism and cell from oxidative DNA damage associated with cancer and degenerative diseases [163,164]. Many *in Vitro* studies evaluated the anticancer properties of both water and alcoholic extracts of *Moringa oleifera* leaves on different types of tumor cells lines. Sreelatha *et al.* [165] found that the aqueous extract of *Moringa oleifera* leaves exhibited a dose-dependent inhibition of cell proliferation of KB human tumor (KB) cells line.*Moringa oleifera* is an important source of food, medicine, therefore, research on antioxidant potential and genetic diversity of this plant will be of great importance in Indian set of conditions because this plant is widely distributed in various eco-climatic zones in India.

Objectives

The present investigation was undertaken to evaluate genetic variations using molecular markers and antioxidant potential of *Moringa oleifera.* The principal objectives of the proposed study are:

1. To evaluate genetic diversity within and among the variants of *Moringa oleifera* in different parts of Rajasthan using RAPD and ISSR markers.
2. To offer genetic resource information for future meaningful implementation of conservation programs applicable for *M. Oleifera.*
3. Spectrophotometric estimation of total phenol and flavonoids from leaves, pods and seeds of both the variants of *M.oleifera* in order to assess the antioxidant diversity among the variants and plant parts.
4. Estimation of antioxidant activity from the extracts of plant materials by DPPH free radical scavenging method for comparative analysis of antioxidant potential in the extracts of plant materials.

5. Review (Work already done on the subject of proposed Research Study.)

Drumstick (*Moringa oleifera* Lam.) is a multi-purpose tropical tree that belongs to Moringaceae family and has originated from Himalayan tract in northwestern part of India (Mendieta-Araica et al., 2012; Pandey et al., 2011). It has diploid chromosome 2n = 28, with some tree flowering throughout the year while others flower in two distinct season (Jyoth et al., 1990; Ramachandran et al., 1980). In India and other countries, immature fruits, fresh leaves and flower of this tree are used for culinary purpose (D'souza and Kulkarni, 1993; Oduro et al., 2008). The *Moringa* seeds were found to exhibit the property of natural coagulants/flocculants, which has potential for use in water and sewage treatment to clear turbidity in drinking water and sludge in sewage respectively (Gassenschmidt et al., 1995; Jahn, 1984; Muyibi and Evison, 1995; Ndabigengesere and Narasiah, 1998). *M. oleifera* seed contains about 35–45% seed oil, having odourless and colourless physical properties (Ayerza, 2012). The edible oil is highly nutritious and is extracted by boiling the seeds with water (Somali et al., 1984). The seed oil has high concentration of oleic acid (>73%) coupled with low polyunsaturated fatty acid, which gives the oil an outstanding and remarkable oxidative stability properties. The suitability of M. oleifera seed oil as biodiesel feed source has been tested by Da Silva et al. (2010) and recommended that, oil could be used as pure biodiesel or petro-diesel mixture on engine after converting it to fatty acid methyl esters (FAME) through the process of trans esterification. Despite the great economic importance, this plant is still unexploited (Pandey et al., 2011). The genetic diversity pattern, genetic makeup and agronomical requirement need to be studied for successful breeding and improvement, domestication and large scale cultivation. The knowledge of genetic diversity of tree crop is very important for rational planning of conventional, modern breeding and improvement programme for the purpose of improving the yield and its quality of produce (Rafii et al., 2012).

The genetic diversity can be assessed based on morphological markers but it is difficult to classify the accessions solely based on their morphological characters therefore the use of molecular markers, such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) has gained popularity as a genetic diversity assessment methods of tree crops (Muluvi et al., 1999; Mgendi et al., 2010; Saini et al., 2013; Tanya et al., 2011; Wang et al., 2012). Assessment of genetic diversity in *M. oleifera* has been done using RAPD (Mgendi et al., 2010; Rufai et al., 2013; Saini et al., 2013), ISSR (Saini et al., 2013) and AFLP markers (Muluvi et al., 1999) in accessions from India as well as Africa. None of the above study has been taken up morphological and SSR markers for diversity study and as we know SSR markers are co-dominant and locus specific hence can give better estimate of genetic diversity in comparison to dominant markers (RAPD, ISSR and AFLP). In the present study an effort has been made to study the genetic diversity of 300 genotypes belonging to 12 natural populations distributed in northern (Himachal Pradesh) and southern part (Tamil Nadu) of India using morphological as well as SSR markers developed by Wu and Yang (2010).

Many *in vitro* studies on antioxidant activity of *Moringa oleifera* leaves are available in literature [56,58,63–65,67,70,115–119]. Siddhuraju and Becker [58] examined the radical scavenging capacities and antioxidant activities of the aqueous, aqueous methanol, and aqueous ethanol extracts of freeze-dried leaves of *Moringa oleifera* from different agro-climatic regions. Moyo *et al.* [122] studied the antioxidant properties of diet supplemented with *Moringa oleifera* dried leaves in goats model. The authors found a significant increment of the activity of GSH in goats supplemented with *Moringa* leaves as compared with the control group.

6. Research Gaps identified in the proposed field of investigation. (Based on the review)

Moringa oleifera is an interesting plant for its use in bioactive compounds. In proposed study concerning the evaluation of antioxidant potential of all the plant parts of *Moringa oleifera* along with genetic diversity among different variants and populations are the major un explored areas of investigation. *Moringa oleifera* shows diversity in many characters and extensive morphological variability, which may provide a resource for its improvement. Great genetic variability is present in the natural and cultivated accessions, but no collection of cultivated and wild accessions currently exists. A germplasm bank encompassing the genetic variability present in *M. oleifera* is needed to perform breeding programmes and develop elite varieties adapted to local conditions. Alimentary and medicinal uses of *Moringa oleifera* are reviewed, alongside the production of biodiesel. Finally, being that the leaves are the most used part of the plant The high contribution in bioactive compounds may explain the pharmacological properties ascribed to *Moringa oleifera* leaves. Many studies conducted on cell lines and animals seem concordant in their support for these properties. However, there are still too few studies on humans to recommend *Moringa* leaves as medication in the prevention or treatment of diseases. Therefore, further studies are urgently needed to explore this plant species thoroughly in Indian context.

7. Major Hypothesis, if any.

In spite of the great variability of *Moringa oleifera* no institutions have a germplasm bank or data base with either cultivated or spontaneous accessions. The divergence between genetic variability inherent to the species and poor variability reflected in germplasm banks should be fixed since it represents an obstacle for the progress of breeding programs.

As a part of an investigation of natural antioxidant of different parts of *Moringa oleifera,* present investigation hypothesize that high antioxidant activity in the plant parts such as leaves, pods and seeds may directly facilitate the pharmaceutical industries for production of large scale bioactive compounds as antioxidant for human welfare.commercial production of drugs to extract antioxidant compounds directly from the cultures/callus of these plant species.

Proposed study further hypothesized that analysis of antioxidant capacity in different parts (Leaves, ripened and un-ripened seeds) of proposed spice plant will ensure the selective utility of these parts as a supplement of antioxidant in Indian foods and vegetables.

8. Research Methodology.

Total phenols determination

Total phenols will be determined by Follin Ciocalteu Reagent method (Mc Donald et al., 2001).An aliquot of each plant extract(0.5ml 1:10 mg/l) or gallic acid (standard phenolic compound) will be added with follin ciocalteu reagent (5ml 1:10 diluted with distilled water) and 4ml of 1M solution of $Na_2 CO_3$ .The mixture will be allowed to stand for 30 minutes at room temperature and absorbance will be measured at 710nm. Total phenolic contents of extracts will be expressed as mg Gallic acid equivalent (GAE)/gm fresh weight. All samples will be analyzed in triplicates.

Total flavonoids determination

Total flavonoid contents will be analyzed by Aluminum Chloride method (Chang et al., 2002). Each plant extract (0.5 ml of 1:10 gm/lt) mixed with 1.5 ml methanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1M potassium acetate and 2.8 ml distilled water. The mixture will be allowed to stand for 30 minutes at room temperature and absorbance will be measured at 415nm.Total flavonoid cotents will expressed as mg quercitin equivalents(QE)/1gm fresh mass. Samples will be analyzed in triplicates.

DPPH- free radical scavenging activity determination

The stable 1,1 diphenyl-2-picryl hydrazyl (DPPH) will be used for *in vitro* determination of free radical scavenging activity of the extracts (Koleva et al., 2002). Different concentrations of each extract will be mixed with methanolic solution of DPPH (0.004%). The mixture will be allowed to stand for 15 minutes. The scavenging of free radicals by extract will be evaluated spectrophotometrically at 517nm against the absorbance of DPPH radicals. The percentage discoloration will be calculated by following formula.

$$\text{DPPH radical scavenging activity (\%)} = \left[AC_{517} - \frac{AE_{517}}{AC_{517}} \right] \times 100$$

Where AC_{517} is absorbance of a DPPH solution without extract, AE_{517} is the absorbance of the tested plant extract with DPPH. The degree of discoloration indicates the free radical scavenging efficiency of the substances. Ascorbic acid will be used as a free radical scavenger reference compound. Values are reported as means ± SD of three determinations.

DNA Extraction

Genomic DNA will be also extracted following the Cetyl Trimethyl Ammonium bromide (CTAB) protocol described by Saghai-Maroof *et al.* (1984) with minor modifications. Leaf tissue (1 g) will be ground to fine powder in liquid nitrogen and transferred to 10 ml 2 X CTAB buffer containing 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB (w/v) and 10 mM β–mercaptoethanol (added freshly). It will be mixed vigorously by vortexing and incubated at 60°C for 30 min followed by treatment with equal volume of chloroform: isoamyl alcohol (24: 1). The upper phase, obtained by centrifugation at 10,000 rpm for 15 min at room temperature, will be transferred to a fresh autoclaved centrifuge tube and to it will be added 1/10 vol 3 M sodium acetate (pH 5.2) and 1/2 vol 5 M NaCl. DNA will be precipitated using 0.6 volume chilled isopropanol and pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant will be decanted and the DNA pellet will be washed twice with 70% ethanol. DNA pellet will be air dried and suspended in 500 μl of 1 X TE.

DNA Purification

Since the DNA contained RNA as impurity, it has to be removed by RNAase treatment (Dellaporta *et al.* 1983). Bovine Pancreatic RNAase (5 mg/ml) will be added (100 μg RNAase/ml DNA suspension) to the dissolved DNA and kept for incubation at 37°C for one hour. To remove the RNAase and other proteins as impurities, an equal volume of chloroform: isoamyl alcohol (24:1) will be added to the DNA solution, mixed by inversion and centrifuged at 10,000 rpm for 15 min at 4°C. The upper aqueous phase will be pipetted out in another MCT using wide bore pipette tips. To it will be added 1/10th volume sodium acetate (3 M) and equal volume of chilled isopropanol will be added and kept at –20°C for one hour. DNA will be pelleted by centrifugation at 10,000 rpm for 10 min at 4°C, washed with 70% ethanol, air dried and dissolved in 100 μl 1 X TE buffer.

DNA Quantification

DNA concentration will be estimated using spectrophotometric method (Samantaray *et al.*2009). Absorbance of the solution will be measured at wavelengths 260 nm and 280 nm. The DNA concentrations will be calculated using following formula:

$$\text{DNA concentration } (\mu g/ml) = \frac{50 \times \ OD_{260} \times \ \text{Dilution Factor}}{1000}$$

The ratio of OD_{260} : OD_{280} will be also calculated. The same procedure will be followed for quantifying all the samples. DNA samples will be diluted to final concentration of 5ng/μl for use in PCR analysis. DNA samples which will have best quality as evident on agarose gel and an OD_{260}/OD_{280} ratio nearer to 1.8 will be used for further analysis.

Optimization of PCR Conditions for RAPD Primers

The PCR conditions for RAPD primers shall be optimized (Ranade *et al.* 2006). The primers will be received as dry desalted powder and a stock solution of 100 pmol μl^{-1} will prepared. Desired amount of elution buffer will be added to the powder and vials will be left over night at 4°C. Finally, a thermal shock at 65°C will be provided for 10 min to ensure complete dissolution of primers. The stock solution shall be used to prepare the working solutions of 10 pmol μl^{-1}.

RAPD primers will be evaluated for their applicability in PCR amplification of total genomic DNA of *Murraya koenigii*. For RAPD analysis fifty-three arbitrary decamer primers (RUF201-RUF220, Sigma Genosys, India and OP1-OP33, Operon Technology, USA) will be used for initial screening and only those primers will be selected for the present study which provided clear and reproducible amplification products under similar conditions.

RAPD profiles shall be produced through PCR amplification using the protocol described by Williams *et al.* (1990) with minor modifications. All the PCR reactions will be carried out (under optimized conditions) in 0.2 ml polypropylene PCR tubes using Thermal Cycler. Each 20 μl reaction mixture contained 1 X Taq buffer (100 mM Tris-Cl (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$ and 0.1% Gelatin), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix (Bangalore Genei, India), 20 pmol oligonucleitide primers (Sigma Genosys, India and Operon Technologies, USA), 1U Taq DNA polymerase (Bangalore Genei, India) and 25 ng template DNA. All reactions will be subjected to initial denaturation at 94°C for 4 min followed by 40 amplification cycles, each consisting of 1 min at 94°C (denaturation step), 1 min at 37°C (annealing step) and 2 min at 72°C (extension step) with a final extension of 7 min at 72°C.

In order to evaluate the effect of different components on DNA amplification, only one component will be varied at a time and other conditions will be kept constant as described in the optimized condition for RAPD.

Optimization of PCR conditions for ISSR

The PCR conditions for ISSR analysis (Verma and Rana, 2011) shall be optimized by taking 20 µl reaction mixture which contains 1 X Taq buffer (100mM Tris-Cl (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$ and 0.1% Gelatin), 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Bangalore Genei, India), 10 pmol oligonucleitide primers (Sigma Genosys, India), 1U Taq DNA polymerase (Bangalore Genei, India) and 25 ng template DNA. Thermal cycler will be programmed with an initial denaturation at 94°C for 5 min, followed by 35 cycles of 30 s denaturation at 94°C, 45 s annealing at respective annealing temperature (45–60°C) and 90 s extensions at 72°C. A final 10 min hold at 72°C ensured full extension of all amplifications products. The amplification products will be stored at 4°C.

In order to evaluate the effect of different annealing temperature on DNA amplification using single primer or pair combination, only one component will be varied at a time keeping other conditions constant as described in the optimized condition for ISSR.

Gel Electrophoresis

Agarose gel preparation

For visualization of genomic DNA 0.8% and for amplified products 1.5% agarose gel will be prepared. Agarose powder will be added to 1 X TAE buffer and boiled in microwave oven. This solution will be cooled to 45–50°C under running tap water. To it added 0.5 µg/ml ethidium bromide will be added, shaken gently and poured into gel casting tray having pre–adjusted comb. The gel will be allowed to set at room temperature.

Electrophoresis

The comb will be carefully removed and the gel casting tray will be placed in submarine gel tank. The gel tank will be then filled with 1 X TAE buffer ensuring that the gel will be completely submerged in the buffer (Ranade *et al.* 2006). Samples will be prepared by taking 4.0 µl of DNA and 1.0 µl of 10 X bromophenol blue dye (0.25% Bromophenol Blue and 50% glycerol) on a Parafilm^{TM} strip and mixed well with the help of a micropipette. PCR products will be electrophoresed in 1 X TAE buffer for ca. 3 h at 80 V on a 1.5% agarose gel matrix. All the amplified products will be separated on 1.5% agarose (w/v) gel, stained with 0.5 µg/ml ethidium bromide. DNA ladder 1 Kb (Bangalore Genei, India) and 100 bp DNA ladder (Bangalore Genei, India) will be mixed and used as molecular weight marker for comparison of amplified products. Gels will be photographed under UV light using a Gel Documentation System (DP 001. FDC, Consort, Belgium). All reactions will be repeated thrice to confirm the results.

Data Analysis

Amplification products will be scored from the gel images as presence or absence of bands (Yeh *et al.*1997). Each band will be treated as one marker. Homology of bands will be based on the distance of migration of amplified DNA fragments according to their molecular weights in the gel. The presence of band will be scored as '1' and absence of band as '0'. DNA polymorphism will be calculated and given as percentage of the total number of bands produced in fingerprinting profiles. All the reactions will be repeated thrice and only those bands which are reproducible for all the runs will be considered for the analysis Co-migrating bands are considered to represent the same locus and thus as the same band while scoring.

9. List of References cited.

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